CONGENITAL ZIKA SYNDROME IN GUINEA PIGS

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Abstract

Zika virus (ZIKV) infection during pregnancy may cause diverse and serious congenital defects in the developing fetus. In this study, we utilized pregnant guinea pigs to study congenital Zika syndrome. Female guinea pigs early in pregnancy (weeks 3–4 of gestation) were inoculated with Asian ZIKV strain (PRVABC59) or PBS (mock) via subcutaneous route. Dams were weighed daily, and blood and urine were collected at regular intervals to assess the presence of virus. Weight loss was observed in ZIKV-infected dams during first week of the infection. ZIKV-infected animals seroconverted with significant viremia and viral secretion in the urine. During the period between infection and delivery of the pups, significant viral RNA and NS1 protein were detected in all animals from 2 to 5 days after infection, with peak viral replication at day 3. We also detected robust viral RNA shedding in urine, with a prolonged duration relative to that of viremia. Dams developed remarkably robust ZIKV-specific neutralizing antibody response, and anti-ZIKV antibodies were also recovered from pups. Notably, ZIKV was efficiently transmitted from infected guinea pigs to their naïve co-caged mates. ZIKV infection of pregnant guinea pigs caused fetal damage. Sixty percent of ZIKV-infected dams showed abnormal pregnancies in that they all delivered at least one or more abnormal pup. Pups from ZIKV-infected animals exhibited significant intrauterine growth retardation. ZIKV was detected in the brain of pups from ZIKV-infected animals. ZIKV RNA and anti-ZIKV antibody levels in the dams reliably predicted abnormalities in pups. ZIKV detection in the brain tissues correlated with the pup abnormalities. In conclusion, the pregnant guinea pig model provides quantifiable

congenital abnormality readouts to assess pregnancy outcomes; and may serve as a good model to test therapeutics, and to elucidate the mechanisms of ZIKV congenital pathogenesis.

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"In my view, all that is necessary for faith is the belief that by doing our best we shall succeed in our aims: the improvement of mankind" – Rosalind Franklin

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Abbreviations

BBB	Blood-Brain Barrier
C	Capsid
CNS	Central Nervous System
CRL	Crown-Rump Length
CZS	Congenital Zika Syndrome
DENV	Dengue Virus
E	Envelope
ER	Endoplasmic Reticulum
FDA	Food and Drug Administration
IACUC	Institutional Animal Care and Use Committee
i-BEC	Induced Brain Endothelial Cell
IFN	Interferon
i-N	Induced Mature Neuron
i-NP	Induced Neural Progenitor
IUGR	Intrauterine Growth Restriction
M	Membrane
NAT	Nucleic Acid Amplification Test
NS	Non-Structural
OF	Occipito-Frontal

PBS	Phosphate-Buffered Saline
prM	Pre-Membrane
PRNT	Plaque Reduction Neutralization Test
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TGN	Trans-Golgi Network
WHO	World Health Organization
WNV	West Nile Virus
YFV	Yellow Fever Virus
ZIKV	Zika Virus

Chapter I.

Background

1.1 Zika Virus

i. History and Epidemiology

Zika Virus (ZIKV) is a mosquito-borne *Flavivirus,* belonging to the family *Flaviviridae.* ZIKV was first discovered in 1947 in Uganda's Zika forest in the blood of a febrile monkey, as well as in crushed suspensions of the *Aedes* mosquito [1]. Following this discovery, only a few human cases appeared, restricted to Africa and Asia. However, in 2007, the first human outbreak of ZIKV occurred in Micronesia, specifically on the island of Yap. The strain that caused this outbreak was the Asian strain. The virus continued to spread across the Pacific Islands, causing another outbreak from 2013-2014 in the French Polynesia. The most recent outbreak began in March 2015 in the state of Rio Grande do Norte, Northeast Brazil. By the end of 2015 there were ~1,300,000 suspected cases in Brazil alone. The virus continued to spread throughout South and Central America, to then reach the lower parts of North America [1]. The United States, including Hawai˙i has confirmed a few travel related cases of ZIKV-associated disease, as well as other countries in Europe [2-4].

Following the outbreak in Brazil, it was apparent that transmission may occur in ways other than by the *Aedes* mosquito [1, 5]. Since ZIKV is a blood-borne pathogen, the virus was found in donor blood, leading to transmission by transfusion [1, 5]. Beginning in February 2016, the Food and Drug Administration (FDA) required that any areas in the United States, as well as Puerto Rico, with ZIKV transmission should stop blood donations, with the exception that the blood bags are tested for the virus using Nucleic Acid Amplification Testing (NAT) or they are treated with pathogen-reduction technology [6].

There have also been multiple cases of sexual transmission from male to female. After returning from trips to areas with ZIKV, the sexual partners who had not been naturally exposed show positive for the virus in their blood [1]. Semen samples of infected males have been tested positive for Zika viral RNA with some cases at levels 100,000 times that of matched blood or urine samples [1, 7]. The viral RNA remains present in the semen for 10 weeks after the onset of Zika-like symptoms [1, 8]. Studies have shown that Zika virus RNA in the semen can persist for much longer periods than other bodily fluids. One man in his 30's became infected and on day 91 after the onset of symptoms, ZIKV was detected in urine, saliva and semen samples using RT-PCR; but on day 134, his semen sample still gave a positive result while the urine and saliva were negative [9].

ZIKV is also transmitted perinatally. A study conducted on 345 pregnant women during the Zika outbreak in Brazil [10] indicated that 53% of the pregnant women were positive for ZIKV by PCR. The pregnancy outcome was available for 125 of these women who were tested positive for ZIKV, with 116 of these women gave live birth and 9 had fetal death. Additionally, there were 5 miscarriages during the first trimester, 2 during the second, and 2 stillbirths during the third trimester. A disproportionally high percentage (9%) of these live births from ZIKV positive mothers were small in size for their gestational age. This may have been due to fetal growth restriction or poor placental perfusion. Additionally, four infants exhibited microcephaly at birth [10].

ii. Virology

ZIKV is part of the *Flavivirus* genus in the *Flaviviridae* family, along with other viruses including West Nile virus (WNV), Dengue virus (DENV) and Yellow Fever virus (YFV) [1, 11]. These viruses are closely related as can be seen in Figure 1, which also shows the various Asian and African strains of ZIKV [12]. Flaviviruses are enveloped viruses, which contain a positive sense single stranded RNA genome that is approximately 11kb in length [1], seen in Figure 3. [13]. The RNA encodes for 3 structural and 7 nonstructural proteins. The structural proteins, the capsid (C), the envelope (E) and the membrane (prM/M) proteins, are integral components of the virion particle, while the nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, have other roles including immune evasion and viral replication [1, 13].

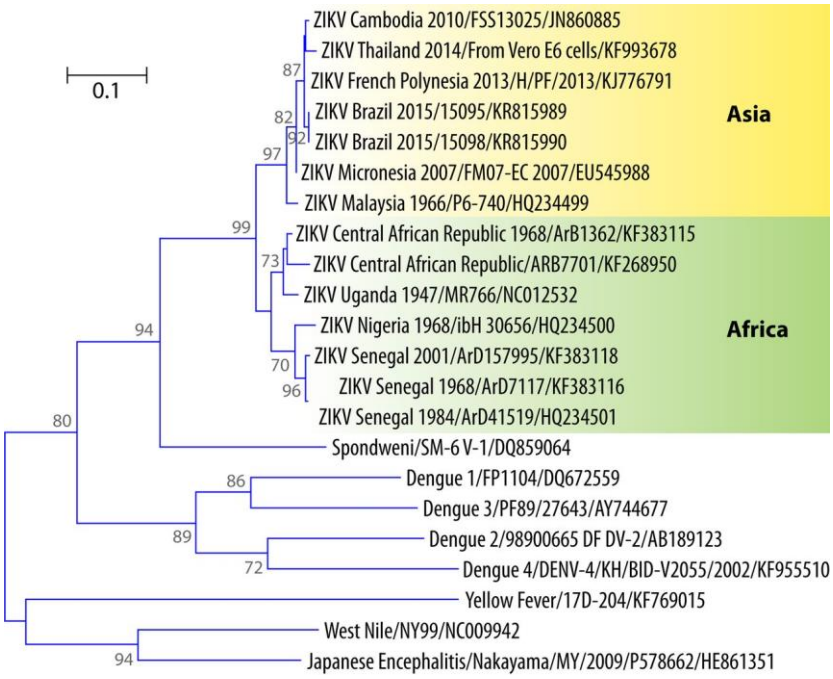

Figure 1. Zika virus phylogenetic tree showing the African and Asian lineages [12].

iii. Life cycle and Replication

ZIKV is an arbovirus, meaning the virus is transmitted primarily through arthropods, including mosquitoes [1]. The mosquito that carries ZIKV is the *Aedes* species, specifically *A. aegypti* and *A. albopictus*, as well as *A. africanus*, which was the first mosquito species in which ZIKV was discovered. There are two life cycles of ZIKV virus, the first being maintained between the mosquito and non-human primates, the sylvatic cycle, and the second, in which humans become infected, the urban cycle [14]. Once the virus is in the salivary glands of the mosquito and the mosquito takes a blood meal from a human, the virus is then transmitted into the blood stream of the human. The cycle may continue by the mosquito taking another blood meal, ingesting the virus, or the virus may be transmitted person-to-person in the ways described before, perinatally, sexually, or by blood transfusion [14].

Following human infection the virus is able to enter cells through receptor-mediated endocytosis and fuses its membrane to the endosome (Figure 2) [14]. The viral RNA is released into the cytoplasm of the host cell due to an acidic-pH-triggered mechanism. Transcription of the positive sense single stranded genomic RNA begins, resulting in the synthesis of the three structural and seven nonstructural proteins. Assembly of the virus takes place at the ER membrane [14], forming immature virions (Figure 3) [13]. The immature virions leave the ER, pass through the Golgi, to reach the trans-Golgi network (TGN) where the acidic pH results in cleavage of the prM protein by the cellular protease, furin. Cleavage of prM results in the dissociation

of the pr segment, generating mature virions that exit the host cell by exocytosis [14]. Many mature viruses are now able to continue infection and replication of new cells.

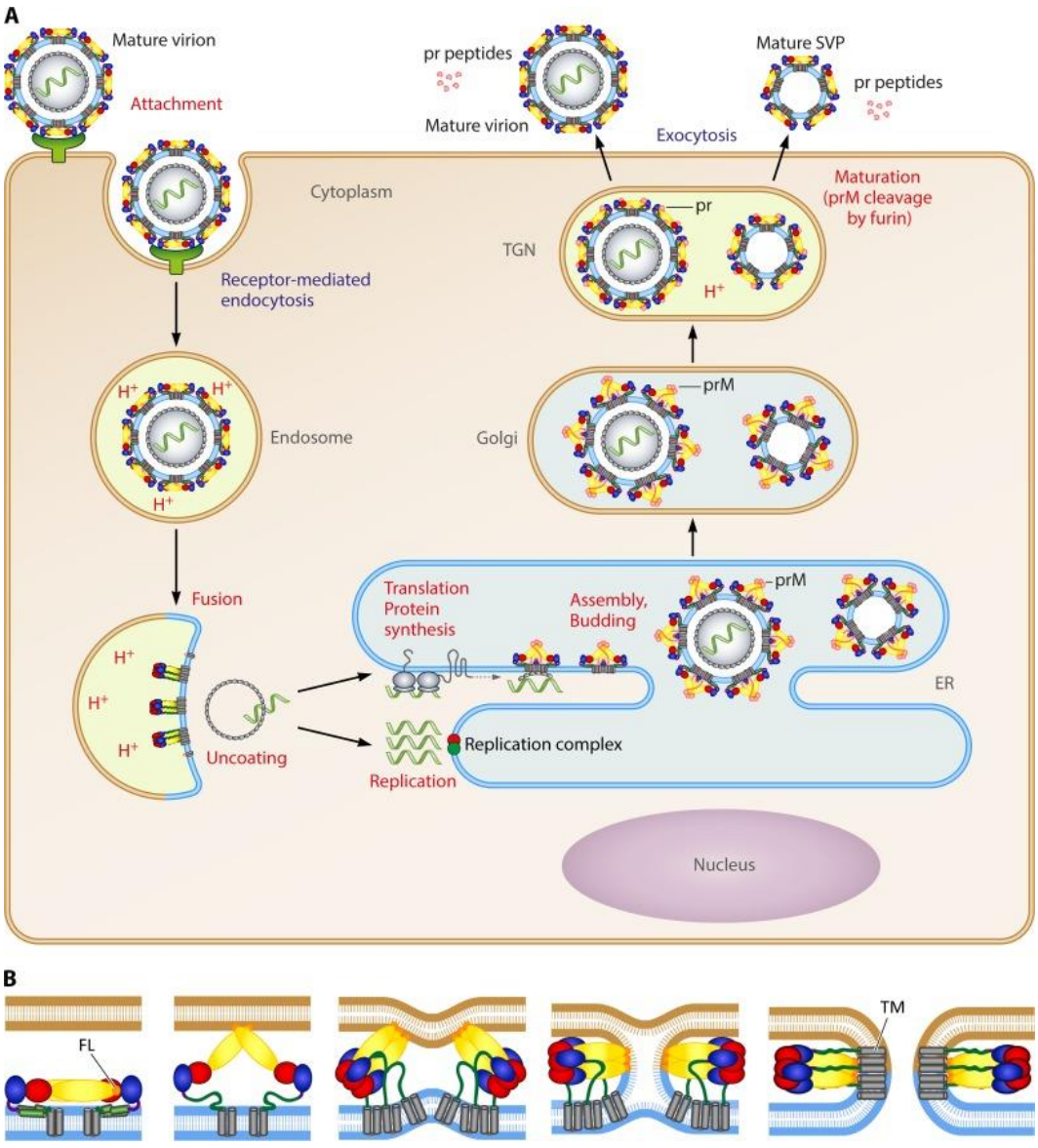

Figure 2. Zika virus replication cycle [15].

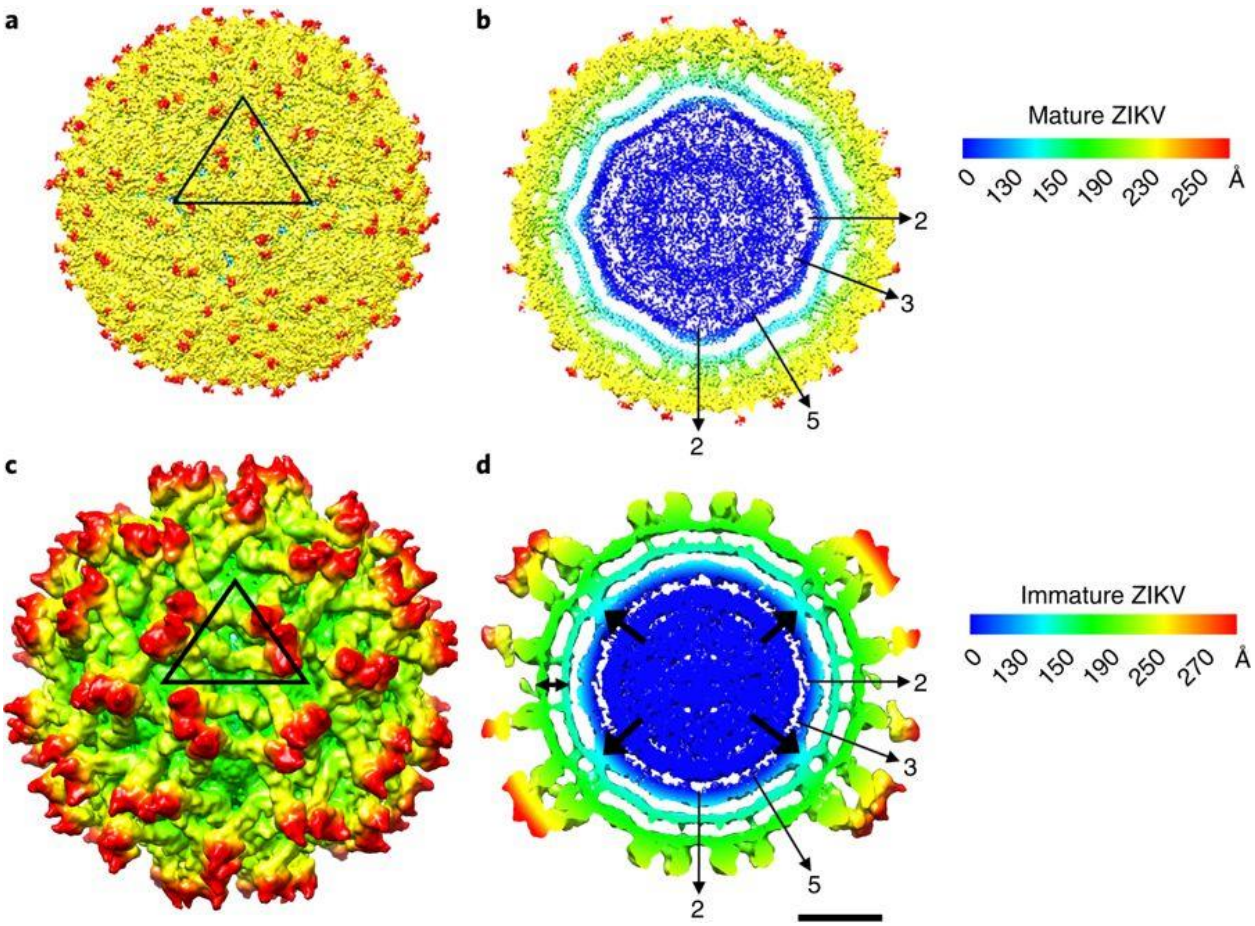

Figure 3. Zika virus structure of mature (top) and immature (bottom) virions [13].

iv. ZIKV Clinical Disease

Approximately 80% of individuals infected with ZIKV are asymptomatic; however, symptoms that may occur include mild febrile illness that is characterized by fever, rash, arthralgia, myalgia, headache, and conjunctivitis [12, 16]. Prior to the 2015-2016 outbreak in the Americas, these were the only known clinical presentations of the disease. However, during these recent outbreaks, there was an increased association between ZIKV infection and fetal abnormalities, such as congenital microcephaly [12, 17, 18]. These fetal abnormalities are what is termed as Congenital Zika Syndrome (CZS). The clinical presentation of CZS includes both structural and

functional components. The structural components include cranial morphology, brain anomalies, ocular anomalies, and congenital contractures, while the functional components are due to neurologic impairments. The underdeveloped brain results in extreme craniofacial disproportioning, including a depression of the frontal and parietal bones collectively known as microcephaly. There has also been evidence of intracranial calcifications, typically in the subcortical region of the brain. This calcification is likely resulted from cell death caused by necrosis or apoptosis. Cell death and cell injury is also the cause of central nervous system damage [18].

v. ZIKV in the brain

The increased association of microcephaly with ZIKV infection of pregnant women is one of the largest concerns of public health officials. Many experiments have been completed to analyze the presence of the virus in the brain, how it crosses the BBB, and which cell type(s) it is infecting. Studies on neonates with CZS [19, 20] and ZIKV infected non-human primate fetuses [21, 22] have observed ZIKV in the brain tissue. An *in vitro* model suggests that the virus is able to cross the BBB through the processes of induced brain endothelial cell (i-BEC) infection, transcellular passage, and subsequent viral release on the other side of the monolayer [23]. The virus then binds to the AXL receptor on cells such as neural progenitor cells (i-NP) and mature neurons (i-Ns). Infection of these cells results in cell death and cell damage, preventing proper development of the brain and central nervous system of fetuses [22].

vi. Therapeutics and Vaccines

Unfortunately, there are currently no licensed therapeutics or vaccines available for ZIKV infection. However, according to the WHO, there are 15 vaccines currently in Phase I clinical trials and one vaccine in Phase II trial [24]. While there is research progress in treatment or prophylaxis, successful development leading to product licensure is still many years away.

2.1 Animal Models

i. Importance of Animal Models

Few animal models of ZIKV infection existed prior to the recent ZIKV epidemics. Only three studies tested the virus' pathogenic potential in animal models between the time of the virus' first isolation in 1947 and the epidemic in 2015 [25-27]. Without the use of a reliable animal model, our understanding of ZIKV infection and pathogenesis, as well as development of vaccines and/or treatments, will be significantly hampered. While there are many animal models available such as mice, non-human primates, and guinea pigs, they each have their advantages and disadvantages. Using the model that mimics human disease the most with as little to no manipulation and is easy and inexpensive to house is ideal.

ii. Mouse Model

In one study, ZIKV was inoculated via intracranial route in mice, causing neurological disease in suckling or adult mice [27]. However, infection with ZIKV via intraperitoneal route in adult immunocompetent mice did not cause disease, indicating that intracranial route of inoculation is necessary for successful infection.

Recently, several groups have also examined immunocompromised adult mice in order to develop a successful mouse model that can support ZIKV replication and disease. Most of these models are deficient in interferon (IFN) response, which is an important component during antiviral defense. These animals developed severe ZIKV disease including paralysis, hind-limb weakness and death after peripheral inoculation. Older immunocompromised mice (11 week-old) are less susceptible to infection than the younger immunocompromised mice (3-5 week-old) [28, 29]. While immunodeficient mice are useful in ZIKV studies, they are inherently biased toward producing disease based on their genetic background. Additionally, lethality is the main endpoint without assessing actual clinical disease.

iii. Non-Human Primate Model

Non-human primates are another common animal model to use due to their anatomical and physiological similarities to humans [30]. This animal model allows for successful ZIKV infection without the use of immunological manipulation. There have been a few studies using rhesus macaques to elucidate ZIKV pathogenesis [20, 21]. However, non-human primates require large facilities that are not readily available to many institutions. Furthermore, the cost of animal purchase and husbandry requirements are significantly higher than those for small animal (rodent) models.

iv. Other Animal Models

Other animal models that have been used for ZIKV studies include hamsters and pigs. Hamsters are similar to mice in that they are small in size and easy to handle. However, in order for ZIKV to cause disease, these animal hosts must be rendered partially

deficient in innate immunity. Accordingly, infection in STAT-2 knockout hamsters results in severe diseases in adult animals and fetuses [31]. While this study was successful in causing disease, using an immunologically manipulated model is not ideal and does not represent natural human infection. Pigs have similar litter size as mice and longer gestational period (approximately 114 day), which allow for adequate sample size and time window for congenital infection studies, such as analyzing CZS [32]. However, similar to non-human primates their housing/husbandry is a challenge in terms of costs and logistics.

v. Guinea Pig Model

An earlier study in 1952 had attempted to inoculate guinea pigs with the African ZIKV strain, MR766 via the intracranial route but the animals developed no signs of infection [27]. However, this strain had previously undergone extensive passaging in suckling mouse brains [33]. Recently, our group showed that guinea pigs are susceptible to infection by a contemporary Asian strain of ZIKV [34]. The guinea pigs were inoculated with the PRVABC59 strain via subcutaneous route resulting in clinical signs of infection including fever, lethargy, hunched back, ruffled fur, and decreased mobility. ZIKV was detected in the serum using qRT-PCR and plaque assays, and infection resulted in a dramatic increase in protein levels of multiple cytokines, chemokines and growth factors in the serum. ZIKV RNA was detected in tissues such as the spleen and brain [34].

Guinea pigs are more physiologically and immunologically similar to humans than other small animals, including mice. Further, guinea pigs are a well-accepted model for congenital infections and sexual transmission [35, 36]. The guinea pig's reproductive physiology and estrous cycle are similar to humans. Also, placentation in the guinea pig

occurs in a similar manner to that of humans, with both guinea pig and human placentas classified as hemomonochorial [37]. Similar to humans, the placenta of guinea pigs has a single trophoblast layer that separates maternal and fetal circulations. They have a long gestation period and pups are born with a mature CNS. These similarities of guinea pigs to humans makes this species a promising animal model to study *in utero* transfer of ZIKV and its neurological manifestations in infants [36]. Similar to mice, guinea pigs are considered a small animal model; however, they do not require immunologic manipulation to establish infection and produce disease.

vi. *In utero* animal models

Table 1. Comparison of *in utero* animal models.

	Guinea Pigs	Mice	Non-Human Primates (Rhesus)	Swine	Hamsters
Cost of purchase	~$100	~$10	~$50,000	~$250	~$50
Size	Small	Small	Large	Large	Small
Gestation period	59-72 days	~20 days	~164 days	~115 days	~22 days
Placenta structure	Hemomonochorial	Hemotrichorial	Hemomonochorial	Epitheliochorial	Hemotrichorial
Does ZIKV cause disease?	Yes	No	Yes	Yes	No

Table 1 demonstrates a comparison of the various animal models commonly used for *in utero* ZIKV research. When comparing the various aspects for each animal model, the guinea pig model looks to be the best to use for *in utero* experiments, especially when infecting with ZIKV.

While the cost of purchase is reasonable for the small animal models, swine are a little more costly, especially if a large cohort is needed, and non-human primates are extremely expensive at a cost of approximately $50,000 for a single rhesus macaque.

The smaller the size of an animal model, the less space they need for housing, which means cheaper daily costs. While guinea pigs are larger than mice and hamsters, they are still considered a small animal model. Non-human primates and swine on the other hand, are large and need a lot of housing space, which most facilities do not have and for those that do, the cost per day to house and care for the animals is quite high.

The gestation period is an important aspect to account for when planning an *in utero* experiment because it can determine how quick the results will be determined, the cost will increase with the longer gestation periods due to the daily husbandry charges. Most importantly, the longer gestation periods corresponds to the stage of development the animals are at, at the time of birth. For mice and hamsters, who have shorter gestation periods, the pups are born without a developed CNS. The longer gestation periods, such as that for guinea pig pups, they will be born with a developed CNS. Animals born without a developed CNS will continue development for about 21 days following birth, delaying some experiments until they are developed enough to examine, as well as not representing human babies who are born with a developed CNS.

Animals vary in their reproductive system, specifically in the structure of their placenta. Humans have a hemomonochorial structure, meaning they have a single trophoblast layer separating the maternal and fetal circulations. Similarly, Guinea pigs and non-human primates also have a hemomonochorial placenta. However, mice and hamsters have a hemotrichorial placenta structure meaning they have three trophoblast layers as opposed

to one. Swine have a different placenta than all of these animal models. They have an epitheliochorial placenta, in which the chorion is next to the lining of the uterus but does not invade or erode the lining. Since humans have a hemomonochorial, guinea pigs and non-human primates would mimic humans for *in utero* experiments.

Since this study is analyzing CZS, ZIKV replication within the animal models is another important aspect to consider. As described above, ZIKV is not able to replicate and cause disease in mice and hamsters. They must first go through immunologic manipulation, which is an additional cost to the project. Studies have shown that the virus is able to replicate and cause disease in guinea pigs, non-human primates, and swine that are not immunologically manipulated.

Chapter II.

Thesis Scope

1.1 Objective, Hypothesis and Rationale:

The ***objective*** of the proposed research is to characterize and utilize the guinea pig model to study *in utero* ZIKV infection. With this model, we will examine the relationship between clinical disease and the derangements observed in the immune system, the nervous system and viral load. Our central ***hypothesis*** is that the guinea pigs will develop ZIKV disease, and transmit virus to fetus after subcutaneous inoculation of ZIKV in mothers.

2.1 Innovation and Significance:

The proposed research is highly ***innovative*** as it will be the first study to employ the guinea pig model to characterize *in utero* transmission and pathogenesis of ZIKV. The findings from this study will have a ***significant impact*** on understanding the mechanisms associated with ZIKV transmission to the fetus, pregnancy outcomes, pathogenesis of sexual transmission, short- and long-term neurological sequelae in infants, and other manifestations in infants including developmental delays and physical disorders. This study will help understand the pathogenic mechanisms underlying the development of ZIKV associated neurological disease. Also, this model system will form the basis to understand the basic biology of ZIKV infection and disease, and to develop strategies to prevent transmission of ZIKV to the fetus, and for evaluating vaccines and therapeutics.

3.1 Specific Aims:

Specific Aim 1:

Inoculate pregnant guinea pigs with ZIKV via subcutaneous route and examine clinical signs and pregnancy outcomes.

Specific Aim 2:

Examine the kinetics of infection and immune response in pregnant guinea pigs.

Specific Aim 3:

Evaluate the presence of ZIKV in the fetuses, and the effects of ZIKV infection on fetal loss, intrauterine development of the fetuses and brain injury.

Chapter III.

Congenital Zika Syndrome in Guinea Pigs

Abstract

Zika virus (ZIKV) infection during pregnancy may cause diverse and serious congenital defects in the developing fetus. In this study, we utilized pregnant guinea pigs to study congenital Zika syndrome. Female guinea pigs early in pregnancy (weeks 3–4 of gestation) were inoculated with Asian ZIKV strain (PRVABC59) or PBS (mock) via subcutaneous route. Dams were weighed daily, and blood and urine were collected at regular intervals to assess the presence of virus. Weight loss was observed in ZIKV-infected dams during the first week of infection. ZIKV-infected animals seroconverted and significant viral secretion in serum and urine was detected. During the period between infection and delivery of the pups, significant viral RNA and NS1 protein were detected in all animals from 2 to 5 days after infection, with peak viral replication at day 3. We also detected robust viral RNA shedding in urine, with a prolonged duration relative to that of viremia. Dams developed remarkably robust ZIKV-specific neutralizing antibody response, and anti-ZIKV antibodies were also detected in pups. Notably, ZIKV was efficiently transmitted from infected guinea pigs to their naïve co-caged mates. ZIKV infection of pregnant guinea pigs caused fetal damage. Sixty percent of ZIKV-infected dams showed abnormal pregnancies in that they all delivered at least one or more abnormal pup. Pups from ZIKV-infected animals exhibited significant intrauterine growth retardations. ZIKV was detected in the brain of pups from ZIKV-infected animals. ZIKV RNA and anti ZIKV-antibody levels in the dams reliably predicted abnormalities in pups. ZIKV detection in the brain tissues correlated with the pup abnormalities. In conclusion, the pregnant guinea pig model provides quantifiable congenital abnormality readouts to assess fetal outcome and may serve as a good model to test therapeutics, and to study the mechanisms of ZIKV congenital pathogenesis.

Background

Zika virus (ZIKV) is a mosquito-borne *Flavivirus,* belonging to the family *Flaviviridae,* along with other viruses including West Nile virus (WNV), Dengue virus (DENV) and Yellow Fever virus (YFV) [1, 2]. ZIKV was first discovered in 1947 in Uganda's Zika forest in the blood of a febrile monkey, as well as in crushed suspensions of the *Aedes* sp. mosquito. Following this discovery, only a few human cases appeared, restricted to Africa and Asia. However, in 2007, the first human outbreak of ZIKV occurred in Micronesia, specifically on the island of Yap. The most recent outbreak began in March 2015 in the state of Rio Grande do Norte, Northeast Brazil. The virus continued to spread throughout South and Central America, to then reach the lower parts of North America [1]. The United States, including Hawai'i, has confirmed a few travel related cases of ZIKV-associated disease, as in several European countries [3-5].

ZIKV is an arbovirus, meaning the virus is transmitted primarily through arthropods, including mosquitoes [6]. However, following the 2015 outbreak in the Americas, there has been evidence of congenital transmission, as well as sexual and blood transfusion transmission [1, 6]. Symptoms of ZIKV may include a "Zika" rash as well as mild flu-like symptoms such as fever, headache, myalgia, and occasionally conjunctivitis [6, 7]. Prior to the 2015 outbreak these symptoms were characteristic of the disease. However, during the outbreak, fetal complications occurred including what is known as Congenital Zika Syndrome (CZS) [6, 8, 9]. If a pregnant woman were to become infected with ZIKV during pregnancy, especially during the first trimester, she has about a 7% chance of having a miscarriage, stillbirth or a baby born with CZS. CZS is characterized by severe microcephaly with partial collapsed skull, decreased brain tissue

and brain damage, ocular scarring and damage, as well as hypertonia restricting body movement soon after birth [9].

Few animal models of ZIKV infection existed prior to the recent ZIKV epidemics. Only three studies tested the virus' pathogenic potential in animal models between the time of the virus' first isolation in 1947 and the 2015 epidemic [10, 11]. These animal models all used mice; however, ZIKV is unable to produce disease in immunocompetent mice. In order to study the evolution of ZIKV infection and pathogenesis, mice must render deficient in one or more components of innate immune responses by genetic ablation, such as the *Ifnar1*$^{-/-}$ knockout mice [12, 13]. Thus, while these types of mouse model are readily available, they are not ideal.

Non-human primates are also a common animal model to use due to their anatomical and physiological similarities to humans [14]. This animal model allows for successful ZIKV infection without the use of immunologic manipulation. There have been a few recent studies using rhesus macaques to better understand ZIKV pathogenesis. However, non-human primates require large facilities that are not readily available at many research institutions. Additionally, the cost of purchase as well as the proper housing requirements are significantly higher than small animal models.

Guinea pigs are more physiologically and immunologically similar to humans than other small animals, including mice. Guinea pigs are a well-accepted model for a number of human infection and disease processes, including congenital infections and sexual transmission [15, 16]. The guinea pig's reproductive physiology and estrous cycle are similar to humans. Also, placentation in the guinea pig occurs in a similar manner to that of humans, and both guinea pig and human placentas are classified as hemomonochorial [17]. Similar to humans, the placenta of guinea pigs has a single trophoblast layer that separates maternal and fetal circulations. They have a long

gestation period and pups are born with a mature CNS. These similarities of guinea pigs to humans make this species a promising surrogate model for studies of *in utero* transfer of ZIKV and neurological manifestations in infants [16].

In an earlier study, inoculation of guinea pigs via the intracranial route with the African ZIKV strain MR 766 produced on infection [11]. However, the strain used had undergone extensive passaging in suckling mouse brains. Recently, it was shown by our group and others that guinea pigs are susceptible to infection by a contemporary Asian strain of ZIKV [18, 19]. The guinea pigs were inoculated with the PRVABC59 strain via the subcutaneous route and results in clinical signs of infection, which include fever, lethargy, hunched back, ruffled fur, and decreased mobility. ZIKV was detected in the serum using qRT-PCR and plaque assays and infection resulted in a dramatic increase in protein levels of multiple cytokines, chemokines and growth factors in the serum. ZIKV RNA was also detected in the spleen and brain [18].

Materials and Methods

Animals: Hartley guinea pigs were purchased from Charles River Laboratory (Wilmington, Massachusetts, United States). This study was approved by the University of Hawaii Institutional Animal Care and Use Committee (IACUC), and conducted in strict accordance with guidelines established by the National Institutes of Health and the University of Hawaii IACUC.

Mating: 3-4 week old guinea pigs were purchased from Charles River. We received 14 males and 14 females of the same age from separate litters to ensure no inbreeding. Once the females were sexually mature one female and one male were housed together to mate. Females were

weighed daily and checked for signs of pregnancy, which included analyzing the vaginal membrane for the mucous plug.

Serum separation: After whole blood collection, the samples were placed at room temperature for 1 hour, then placed on ice. The samples were then centrifuged for 15 minutes at 10,000 rpm to allow separation of the serum. Serum was then aliquoted and stored at -80°C until needed for experiments.

Progesterone ELISA: After one week of pairing, 25 μL of serum from each female on a weekly interval was used to analyze progesterone levels. The Human Progesterone ELISA Kits used for this analysis were purchased from DRG (Cat# EIA1561). Once the progesterone levels reached above 20 ng/mL, the female guinea pigs were confirmed pregnant.

ZIKV Infection: ZIKV strain PRVABC59 (BEI Resources) was used in this study. At 3-4 weeks after conception, nine female guinea pigs were subcutaneously inoculated with either $1x10^6$ PFU sequence-verified Puerto Rico ZIKV strain and five were inoculated with PBS as the mock infection group. Following infection, guinea pigs were observed daily for the next 7 days, then weekly until birth, for signs of infection. Clinical signs included ruffled fur, hunched back, and skin tone. Body temperature was taken for signs of fever, and blood was collected to assess the presence of viremia and anti-ZIKV antibodies.

NS1 ELISA: The presence of the NS1 protein in guinea pig serum samples was analyzed using the Zika virus NS1 ELISA kit (BioFront Technologies) following the manufacturer's protocol (Cat# ZIKV-NS1-EK-96).

RNA Extraction: Total RNA from guinea pig tissues was extracted using the RNeasy Mini Kit (Qiagen) following manufacturer's protocol (Cat# 74106). About 200 mg of frozen tissue (brain,

spleen, kidney, and liver) was powdered over dry ice to obtain homogenous samples for total RNA extraction.

Synthesis of cDNA from cellular RNA: One microgram of cDNA was synthesized from 1 μg of cellular RNA using iScript Select cDNA Synthesis Kit (Bio-Rad Cat# 170-8896) following manufacturer's procedures. In brief, reaction mix, reverse transcriptase and RNA template were mixed to a final reaction volume of 20 μL by adding nuclease-free water. The reaction mixtures were incubated at 42°C for 45 min, followed by 85°C for 5 min to heat-inactivate the reverse transcriptase.

Table 1. Primer sequences used for qRT-PCR

Gene	Primer sequence (5'-3')	Amplicon (bp)	Tm (°C)
ZIKV Primer			
Forward 1086	CCGCTCCCCAACACAAG	17	57.4
Reverse 1162c	CCACTAACGTTCTTTTGCAGACAT	24	55.8

qRT-PCR: Fold-changes in the ZIKV viral RNA were determined by real-time qRT-PCR. Less than 1000 ng (maximum amount stated by manufacturer's protocol) of cDNA was used as template for real-time PCR assays with the ZIKV primers listed in Table 1. Standard curves were generated using RNA extracted from previously titrated ZIKV dilutions. The cDNA templates and the primers were combined with the iQ Supermix (Bio-Rad Cat# 1725160) and used for PCR. PCR thermal cycling was initiated with a first denaturation step of 95°C for 15 min followed by 40 cycles of 94°C for 15 sec and 60°C for 1 min, and then brought down to 4°C for 5 min.

Plaque Reduction Neutralization Test (PRNT): The serum titers of anti-ZIKV neutralizing antibodies were measured using the PRNT as described previously [20, 21]. Serum was collected from the dams daily for the first week after infection followed by weekly until the time of birth. Serum was also collected from the naïve co-caged mates, as well as the pups, at the time of euthanasia. The serum samples were diluted from 1:20 to 1:20,480 and PRNT was conducted against ZIKV PRVABC59 strain. The highest dilution of serum resulting in 50% reduction in the number of plaques compared to the growth of the virus control was determined as described previously [21].

ZIKV detection in pups brain using virus culture: About 150 mg of frozen brain tissue was homogenized using the bullet blender method. An equal amount of 0.5mm Zirconium beads was added to the tissue sample together with protease inhibitors. The mixture was placed in the bullet blender for 3 minutes at Speed 6. A 100 μL of the homogenate was used to infect Vero cells. The cell lysate and its supernatant were harvested 5 days later. The cell lysate was then used for qRT-PCR while 100 μL of the supernatant was used for a second round of infection with fresh Vero cell cultures. Following the second round of infection, the cell lysate was collected once again and analyzed by qRT-PCR.

Guinea pig pup size measurements: Within the first 24 hours after birth, the pups were weighed and measured to evaluate morphologically. The measurements that were taken included crow-rump length (CRL), indicated by the blue line; occipitofrontal (OF) diameter, indicated by the green line; and the head circumference, indicated by the purple line. The CRL and OF measurements were then multiplied to determine the overall pup size.

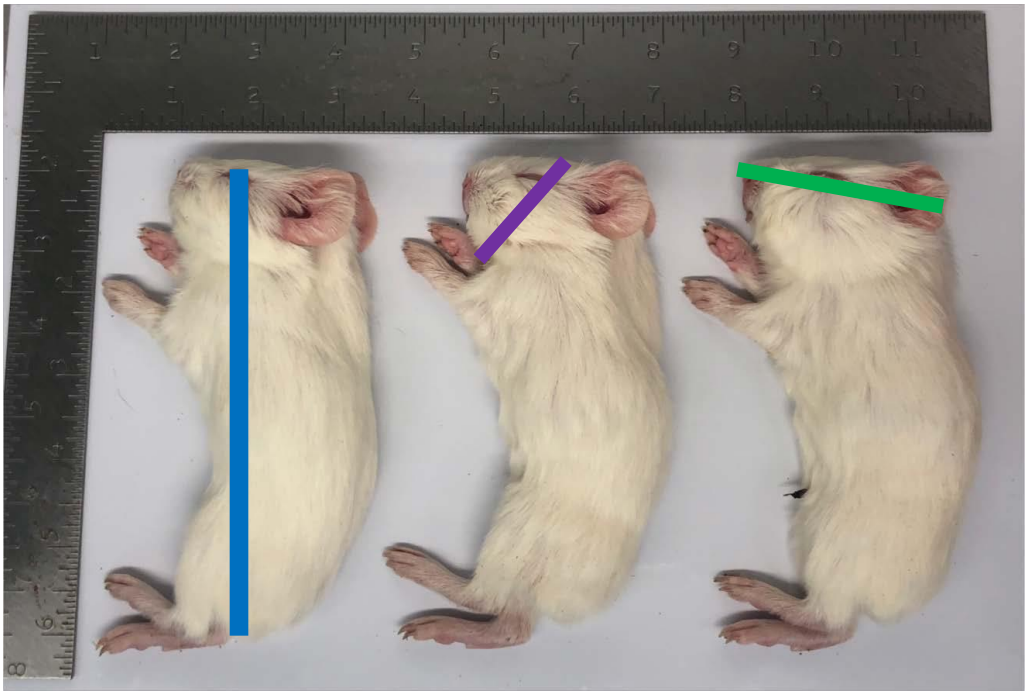

Figure 1. Guinea pig pup measurements. Overall body size measured by the crown-rump length (blue) and the occipito-frontal diameter (green). The head circumference was measured using the diameter (purple).

Results

Pregnant guinea pigs are susceptible to ZIKV infection

To determine whether ZIKV is infectious in guinea pigs, time-mated pregnant guinea pigs were infected with ZIKV or PBS (Mock). Nine dams were subcutaneously inoculated with 10^6 plaque-forming units (PFU) of Asian ZIKV strain (PRVABC59). Five dams were inoculated with PBS (Mock). The guinea pigs were inoculated at 3-4 weeks of gestational age, equivalent to the end of the first trimester. Every day for the first week, followed by weekly until the end of the pregnancy, the guinea pigs were weighed and observed for clinical signs of disease including ruffled fur, hunched back, fever and weight loss.

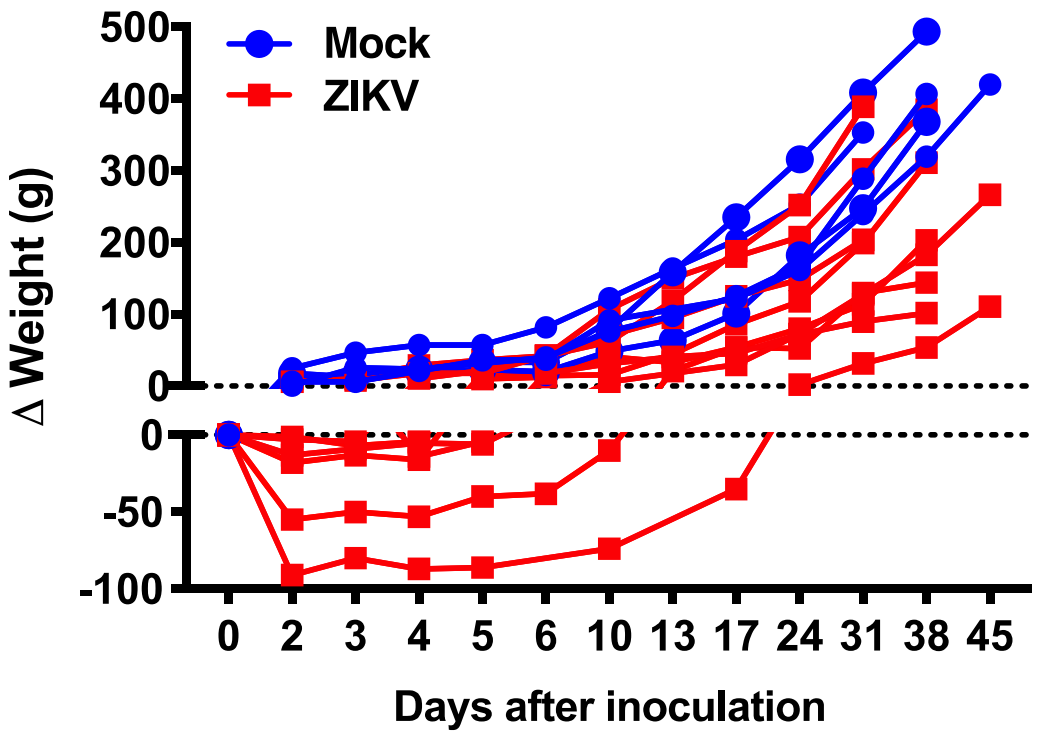

Figure 2. Weights of pregnant guinea pigs. Following infection, pregnant guinea pigs were weighed once a day for 1 week followed by once a week until they gave birth. Control guinea pigs (blue) showed a steady weight gain. ZIKV infected guinea pigs (red) showed a decrease during the first week after infection.

All five pregnancies of mock-infected dams were normal. Five out of nine pregnancies were adversely affected after ZIKV infection, while four pregnancies were normal. Mock-infected dams gained between 400-500 g weight during the pregnancy (Figure 2). Weight loss was observed in five out of nine ZIKV-infected dams during the first week of infection. Total weight gain of these five ZIKV-infected dams during the pregnancy was only between 100-200g. The other four ZIKV-infected dams did not lose any significant weight during the first week of

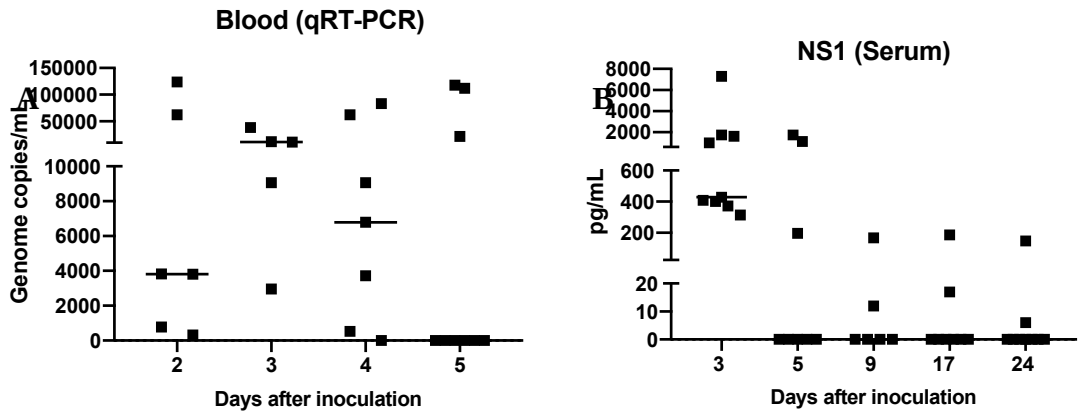

Figure 3. ZIKV detection in dams. (A) All infected female guinea pigs showed significant viral RNA in the whole blood at days 2 to 5 after infection as measured by qRT-PCR. (B) Circulating NS1 protein detected in the serum of infected guinea pigs using ELISA.

infection. Total weight gain in these four ZIKV-infected dams was similar to the mock-infected dams. No other clinical signs of illness, ruffled fur, hunched back, or fever, were observed in ZIKV-infected dams. Also, none of the infected dams showed clinical signs that met humane endpoints or spontaneously died of infection.

Kinetics of infection and antibody response in pregnant dams

Whole blood and serum were collected at regular intervals to assess the presence of viral RNA (Figure 3A), NS1 protein (Figure 3B), and anti-ZIKV antibodies (Figure 4). ZIKV-infected pregnant guinea pigs exhibit seroconversion and significant viral replication in the serum. ZIKV-specific qRT-PCR detected viral RNA in the whole blood of all nine infected dams. Viral RNA was detected in all animals from 2 to 4 days after infection, with peak titers of 4.5 to 5.5 log RNA copies/mL at day 3 after infection. Only three infected dams had detectable viremia at day 5. We also measured circulating levels of ZIKV NS1 protein in serum using ELISA. NS1 protein

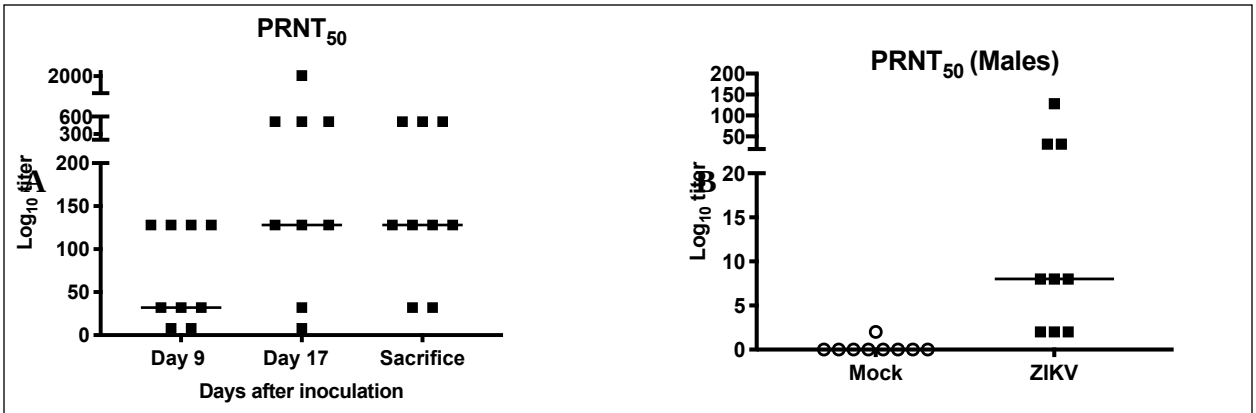

Figure 4. ZIKV-specific antibody levels detected in dams and male guinea pigs using PRNT. ZIKV-specific antibodies detected in (A) infected dams beginning at day 9 after infection until the time of euthanasia, as well as, (B) the co-caged males at the time of euthanasia.

was detected in all nine animals at day 3 after inoculation. Similar to RNA, only three animals had detectable levels of NS1 at day 5. Two animals continued to have low levels of detectable NS1 until day 24 after inoculation. Notably, high and persistent viremia was observed in the dams that lost significant weight and had abnormal pregnancies. ZIKV was not detected in the serum of mock-infected animals.

We next measured anti-ZIKV neutralizing antibody titer in the serum of infected animals using plaque reduction neutralization test (PRNT). Anti-ZIKV neutralizing antibodies were not detected in the serum of all five mock-infected dams. Infected dams developed a remarkably robust ZIKV-specific neutralizing antibody response as early as day 9 after infection and sustained thereafter (Figure 4a). Similar to the viremia, high antibody titers were observed in the dams that lost significant weight and had abnormal pregnancies. ZIKV was efficiently transmitted from infected female guinea pigs to naïve co-caged male animals (Figure 4b). All contact guinea pigs exhibited seroconversion.

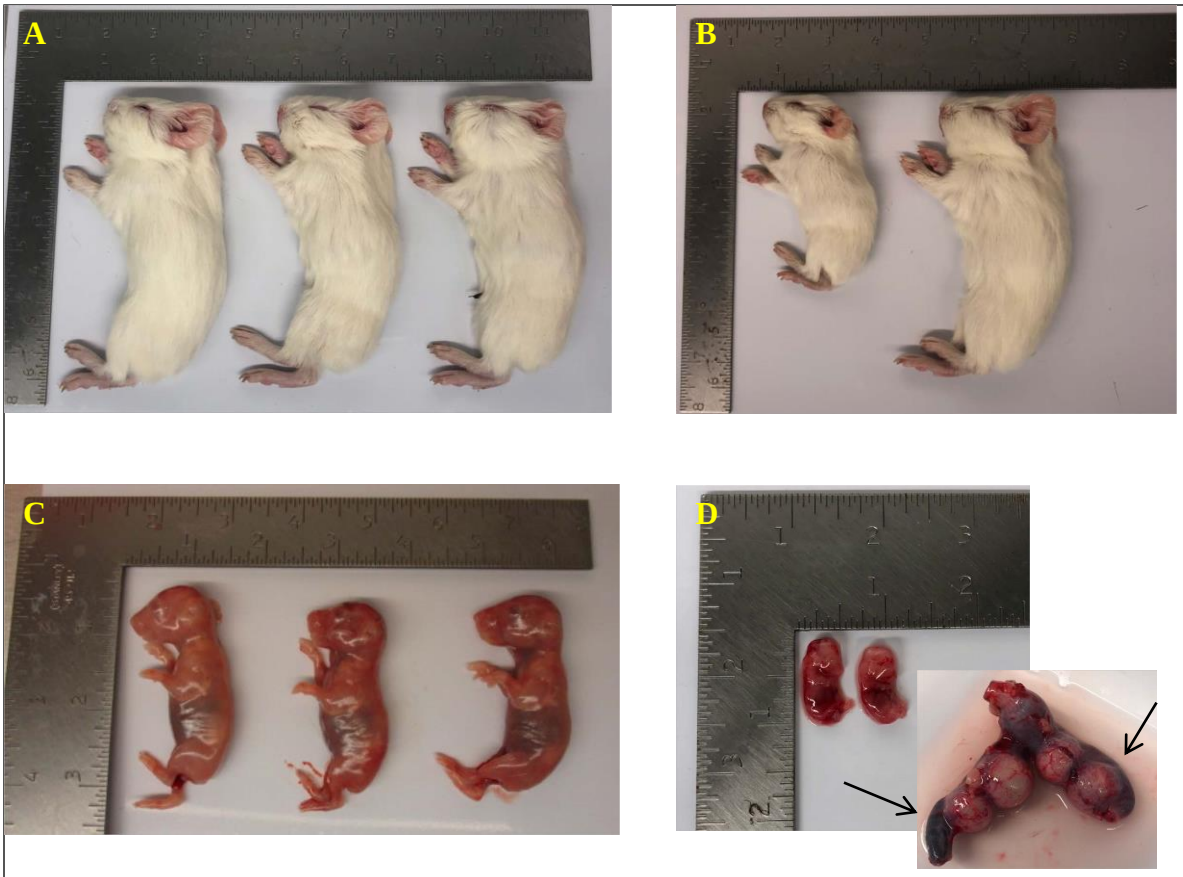

Figure 5. Morphological evaluation of guinea pig pups within 24 hours of birth. (A) Mock pups all showed similar size in both CRL length and OF diameter. (B) Pups born to infected dams showed a variation in both CRL length and OF diameter. (C-D) Pups collected from dams euthanized due to excessive bleeding. Black arrows indicate necrosis due to reabsorption of fetuses.

Congenital Zika syndrome in guinea pigs

Within the first 24 hours of birth, individual fetuses were evaluated for morphological abnormalities, including weight, body size and head circumference by measuring the crown-rump length (CRL) and the occipito-frontal (OF) diameter of the fetal head (Figures 5 and 6). 13 pups born to ZIKV-infected dams were normal, while 10 pups were adversely affected. One ZIKV-infected dam delivered a litter of dead pups around the time of her expected due date

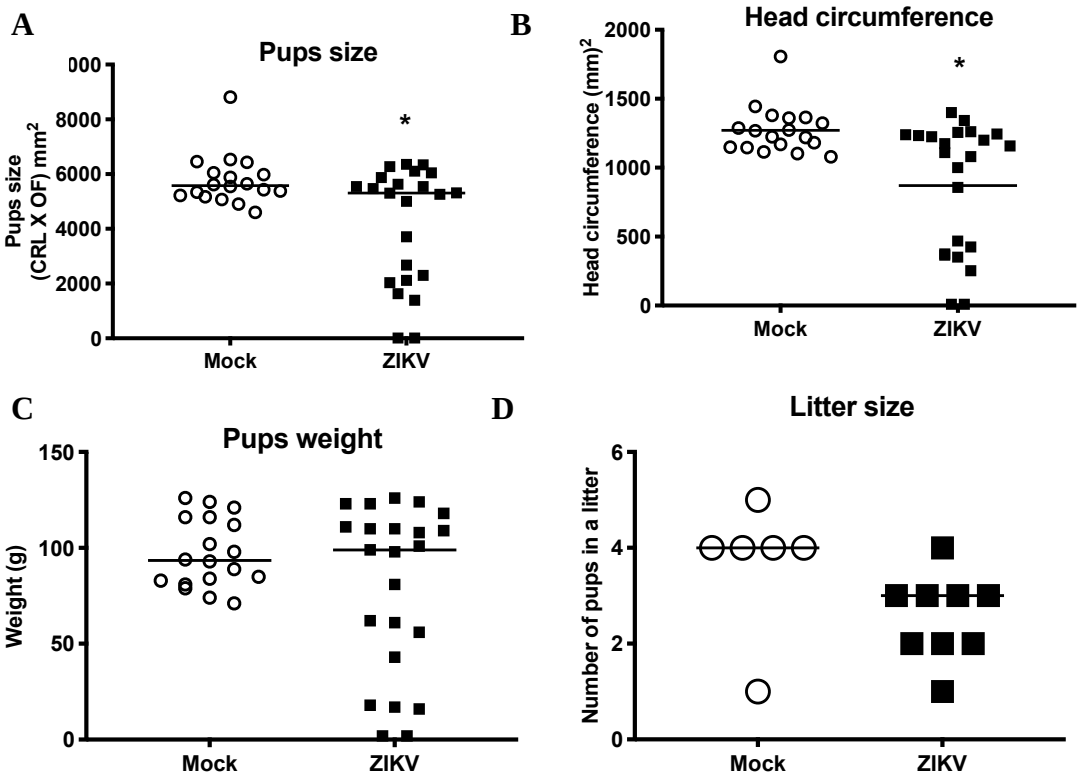

Figure 6. Guinea pig pup measurements. Pups born to dams infected with ZIKV show significant reduction in (A) size and (B) head circumference. (C) There was a trend towards lower weight in ZIKV-infected pups. (D) There were fewer pups per animal in ZIKV-infected cohort than mock-infected cohort.

(Figure 5C). Another ZIKV-infected dams had profuse bleeding two weeks before her expected due date and was euthanized (Figure 5D). This dam began to reabsorb some fetuses, resulting in tissue necrosis as seen in figure 5D. Progesterone levels were measured throughout the pregnancy and both females showed elevated levels until the time of euthanasia. Overall, pups from ZIKV-infected dams were smaller than their mock-infected counterparts. Pups from ZIKV-infected dams exhibited significant intrauterine growth restriction (IUGR).

Guinea pig pups born to ZIKV-infected females exhibited intrauterine growth restriction

Along with analyzing the pups for morphological abnormalities, measurements were taken including pup size (Figure 6A), head circumference (Figure 6B), and weight (Figure 6C). When

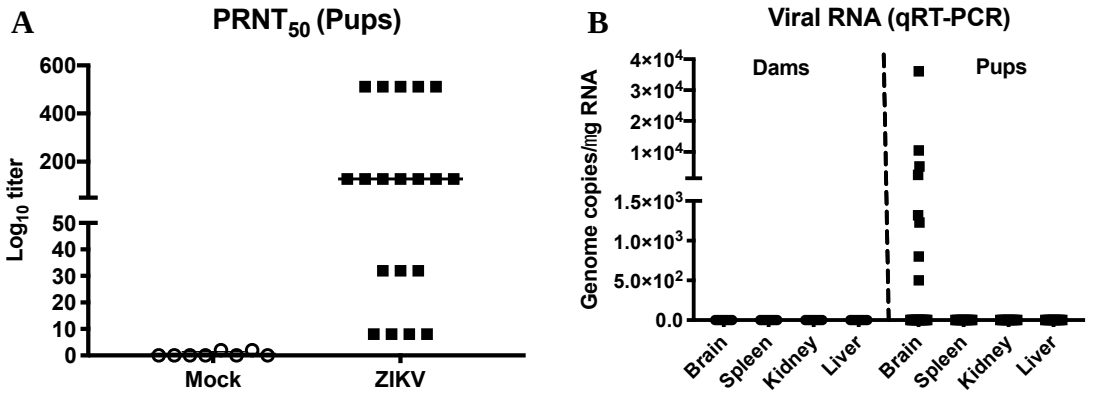

Figure 7. ZIKV-specific antibody and ZIKV viral RNA detected in pups born to ZIKV infected dams. (A) ZIKV-specific antibodies were detected in all pups born to ZIKV infected dams at the time of birth using PRNT. (B) ZIKV viral RNA was detected in the brains of the pups born to ZIKV infected dams using qRT-PCR.

looking at overall size using the crown-rump length and the occipito-frontal diameter, there was a decrease in size of the pups born to infected dams, as well as head circumference. The head circumference is significantly decreased compared to the mock. Similarly, there was a trend towards lower weight in ZIKV-infected pups, but it was not statistically significant. One aspect to take into consideration when looking at pup size is the litter size (Figure 6D). The smaller the litter the more room the fetuses have to grow because the space is not as restricted. However, when comparing the litter size to the pup size it was noticed that the litters born to the infected females were on average smaller than the control females, as well as the pup size. Overall, there are fewer pups per animal in ZIKV-infected cohort than mock-infected cohort and the sizes of the pups born to infected females were significantly smaller. The pups that exhibited the smallest sizes and IUGR were born to ZIKV infected females who had the most weight loss, the highest levels of both circulating ZIKV RNA and NS1 protein, as well as the highest levels of ZIKV-specific antibodies.

Table 2. ZIKV detection in pups brain using virus culture. Infectious viral RNA was recovered from brain tissue of more than 50% of guinea pig pups born to infected dams while there was not any viral RNA recovered from the pups born to mock dams.

	First infection (CT value)	Re-infection (CT value)	Result
Mock			
GP 7a brain	37.42	40.8	Negative
GP 7b brain	36.5	38.37	Negative
GP 31a brain	34.8	39.8	Negative
ZIKV			
GP2a	41.07	32.31	**Positive**
GP2b	23.26	11.39	**Positive**
GP2c	35.42	33.19	Negative
GP5a	31.79	24.53	**Positive**
GP8a	37.57	28.14	**Positive**
GP8b	38.16	32.04	**Positive**
GP6a	38.92	34.41	Negative
GP6b	41.82	30.58	**Positive**
GP9a	41.6	31.56	**Positive**
GP 9b	36.22	38.27	Negative
GP 9c	35.43	36.65	Negative

ZIKV-specific antibodies and viral load in pups

At the time of euthanasia, blood was collected from the guinea pig pups to analyze for ZIKV-specific antibodies. Anti-ZIKV antibodies were also detected in all of the pups from the ZIKV challenged dams (Figure 7A), suggesting that maternal antibodies to ZIKV are transmitted transplacentally. The pups with the highest levels of antibodies were born to the females with the weight loss, and showed high viremia and high antibody production.

ZIKV RNA was analyzed from the brains of the pups born to infected females (Figure 7B). Other tissues such as spleen, kidney, and liver were also analyzed; however, ZIKV RNA was

detected only in the brain tissue. Genome copies ranged from 5.0 x 10^2 copies/μg to 4.0 x 10^4 copies/μg in 50% of the pups born to ZIKV-infected females. The pups with the highest levels were born to the females with the weight loss at the time of infection, as well as the increased levels of ZIKV RNA, NS1 protein, and ZIKV-specific antibodies in the blood. The tissues from female guinea pigs were also analyzed for ZIKV RNA, however, no RNA was detected. Since guinea pigs have a long gestation period, about 40 days, ZIKV infection may have resolved prior to birth. We also conducted viral detection on Vero cells using pups brain homogenates, and the results showed that infectious ZIKV were directly recovered from eight out of twenty-three brain samples.

ZIKV detection in pups brain using virus culture

Since viral RNA was found in the brains of guinea pig pups using qRT-PCR, a virus detection was completed using brain homogenate and vero cells (Table 2). The CT values are compared to a standard curve of previously titrated ZIKV dilutions. ZIKV was directly recovered from more than 50% of the pups. The pups born to mock dams did not have any viral RNA detected. The results show that not all pups in the same litter had ZIKV RNA recovered from the brain tissue, which is consistent with what has been observed throughout the study.

Discussion

Prior to the 2015 ZIKV outbreak in Brazil, as well as other areas in Central and South America, only a few animal studies with ZIKV were completed, and all used mice as animal model [10, 11]. While mice are generally a good model to use due to their small size, they may not be an ideal animal to study ZIKV infections since the virus cannot cause disease unless a genetic

knockout mouse model is used. Following the outbreak, there was an increased need for animal research, and animal models that do not require manipulation and resemble human infection were in demand. The pregnant guinea pig model used in this study provides easily measurable congenital abnormality readout to assess fetal outcome, and for studying the mechanisms of ZIKV congenital pathogenesis, all without having the need to manipulate the host's immunological status.

Previous studies using the guinea pig model reported clinical signs, such as fever, ruffled fur, and hunched back after infection with the Asian strain of ZIKV [18, 19]. Our study did not observe these same clinical signs. Kumar et al. infected the guinea pigs through subcutaneous route; however, the guinea pigs were at a younger age (5 weeks) than in this experiment (about 10 weeks). Age is important for experimental design for a number of reasons. Studies have shown that young animals are still developing, making them more susceptible to infection, than their middle-age counterparts [22-25]. Even though our ZIKV infected guinea pigs did not show the same clinical signs as previously exhibited, four of the dams did lose weight as compared to the mock and the five additional ZIKV infected dams. The dams with weight loss exhibited the highest levels of ZIKV RNA in whole blood, NS1 protein in the serum, and a robust ZIKV-specific antibody production. Following birth, the pups that exhibited abnormalities were born to the dams who had lost weight after infection.

Pregnancy outcomes following ZIKV infection have been reported with various abnormalities including miscarriage, fetal loss, stillbirths, small size for gestational age and microcephaly [26, 27]. ZIKV infection of pregnant guinea pigs results in a fetal syndrome that resembles the intrauterine growth restriction (IUGR) observed in ZIKV-infected pregnant women. ZIKV RNA

and anti ZIKV-antibody levels in the dam reliably predicted fetal abnormalities since the pups exhibiting IUGR were born to the dams most affected by ZIKV infection. Anti-ZIKV antibodies detected in all of the pups from the ZIKV infected dams suggest that maternal antibodies to ZIKV are transmitted transplacentally. Viral RNA in brain tissue and from virus culture was detected in around 50% of pups, correlating with the fetal abnormalities. Since the pups that exhibited IUGR were born to the dams most affected by the ZIKV, we are able to predict the outcome of the pregnancy based on the dam's status.

The male guinea pigs that were housed with infected female guinea pigs developed a robust amount of ZIKV-specific neutralizing antibodies. It was not determined at what point or through which route the virus was transmitted but at the time of euthanasia they ZIKV antibodies were detected in the serum. Since the females have the vaginal plug and do not allow intercourse during pregnancy, sexual transmission is not thought to be the route. Urine and saliva were also collected and ZIKV was detected in the urine of the females by RT-PCR during the first week after infection. The male guinea pigs were observed ingesting and being exposed to the urine, causing us to suspect that this was the route of transmission.

This small animal model for ZIKV may serve as a good model to test prophylactic and therapeutic interventions to protect the fetus during pregnancy, as well as to study related flaviviruses such as dengue virus.

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Chapter IV.

Future Directions

4.1 **Summary:**

Zika virus (ZIKV) is an arbovirus that has been known to cause a “Zika” rash, along with mild flu-like symptoms. While the virus was not a major concern for many decades, with the recent 2015 outbreak in the America’s, there was an association of ZIKV and microcephaly, as well as other birth defects. It is with this association that the virus became a public health concern, due to the lack of prophylactic and therapeutic interventions, as well as a lack of research on the pathogenesis of the virus. Only a few experiments had been completed prior to the outbreak using mice as animal models. However, mice are not ideal due to the immunologic manipulation required for the virus to produce disease.

A need for an animal model that represents human pathogenesis sparked many experiments analyzing various genetic knockouts of mice and hamsters, as well as the use of non-human primates and pigs. However, these animal models also have shortcomings.

Previous studies using the guinea pig model reported clinical signs, such as fever, ruffled fur, and hunched back after infection with the Asian strain of ZIKV [34, 38]. Our study did not observe these same clinical signs. Kumar et al. infected the guinea pigs through subcutaneous route; however, the guinea pigs were at a younger age (5 weeks) than in this experiment (about 10 weeks). Age is important for experimental design for a number of reasons. Studies have shown that young animals are still developing, making them more susceptible to infection, than their middle-age counterparts [41-44]. Even though our ZIKV infected guinea pigs did not show the same clinical signs as previously exhibited, four of the dams did lose weight as compared to the mock and the five additional ZIKV infected dams. The dams with weight loss exhibited the highest levels of ZIKV RNA in whole blood, NS1 protein in the serum, and a robust ZIKV-

specific antibody production. Following birth, the pups that exhibited abnormalities were born to the dams who had lost weight after infection.

Pregnancy outcomes following ZIKV infection have been reported with various abnormalities including miscarriage, fetal loss, stillbirths, small size for gestational age and microcephaly [10, 45]. ZIKV infection of pregnant guinea pigs results in a fetal syndrome that resembles the intrauterine growth restriction (IUGR) observed in ZIKV-infected pregnant women. ZIKV RNA and anti ZIKV-antibody levels in the dam reliably predicted fetal abnormalities since the pups exhibiting IUGR were born to the dams most affected by ZIKV infection. Anti-ZIKV antibodies detected in all of the pups from the ZIKV infected dams suggest that maternal antibodies to ZIKV are transmitted transplacentally. Viral RNA in brain tissue and from virus culture was detected in around 50% of pups, correlating with the fetal abnormalities. Since the pups that exhibited IUGR were born to the dams most affected by the ZIKV, we are able to predict the outcome of the pregnancy based on the dam's experimental results.

The male guinea pigs that were housed with infected female guinea pigs developed a robust amount of ZIKV-specific neutralizing antibodies. It was not determined at what point or through which route the virus was transmitted but at the time of euthanasia they had a continued production of antibodies. Since the females have the vaginal plug and do not allow intercourse during pregnancy, sexual transmission is not thought to be the route. Urine and saliva were also collected and ZIKV was detected in the urine of the females by RT-PCR during the first week after infection. The male guinea pigs were observed licking and being exposed to the urine, suggesting the route of transmission.

It is with our guinea pig experiment that we have found an animal model that represents human pathogenesis with no immunologic manipulation, that is small, and relatively inexpensive to house and care for the animals.

4.2 **Future Directions:**

The immunologically unmanipulated pregnant guinea pig model of ZIKV infection provides easily measurable congenital abnormality readout to assess fetal outcome. Future studies using this animal model should enable testing of prophylactic and therapeutic interventions to protect the fetus during pregnancy, as well as studying the mechanisms of ZIKV congenital pathogenesis. The next important step is to elucidate the immunological mechanisms of disease pathogenesis. Now that we know the virus causes disease in guinea pigs and where the virus may be found, it is important to examine how the virus is causing the disease, which cell receptors it uses to gain access on the various cell types, and how the immune system function to neutralize the virus. Observational analysis of guinea pig pup development following birth may help to understand the effects of disease of children born to infected pregnant mothers.

Further questions may be asked from this project such if antibody levels in the dams correlate with microcephaly and IUGR. If the antibody response is poor and not protecting efficiently against the virus it has potential to cause a more serious outcome. This could help predict the child's outcome prior to birth based on the antibody response produced by the mothers who had been infected during pregnancy.

Insights gained from these studies should facilitate the development of vaccines and anti-virals. Such prophylactic and therapeutic interventions are urgently needed due to the significant

disease complications, such as Congenital Zika Syndrome, caused by ZIKV as discovered from the 2015 outbreak in the America's.

Furthermore, this animal model may be used for other closely related flaviviruses, such as dengue virus. There have been recent experiments examining the relation between dengue virus and Zika virus in terms of severity of disease, as well as association with Congenital Zika Syndrome. The guinea pig model would provide an unmanipulated model to represent human pathogenesis to determine the relation between these two flaviviruses.

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